Kidney International, Vol. 46 (1994), pp. 1564–1566

Transcription factors modulating angiotensinogen gene expression in hepatocytes

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Transcripting factors modulating angiotensinogen gene expression in hepatocytes. The gene encoding angiotensinogen is regulated at the transcriptional level in hepatocytes in response to glucocorticoids and inflammatory cytokines (IL-1 and TNF). These hormones activate transcription of the angiotensinogen gene by changing the abundance of DNA binding proteins that interact with a multihormone-inducible enhancer located between nucleotides −615 to −440 upstream of the major transcription start site. Activation of this enhancer in hepatocytes is effected by glucocorticoid- and cytokine-inducible DNA binding proteins. Cytokine induction is mediated through the interaction of two classes of transcription factors that bind to the acute-phase response element (APRE): nuclear factor-κB (NF-κB), and CCAAT-Box/Enhancer Binding Protein (C/EBP). NF-κB is a multiprotein DNA binding complex sequestered in the cytoplasm that is induced in the nucleus by cytokines, whereas C/EBP is a nuclear transcription factor family implicated in the expression of differentiated hepatic proteins. During the acute-phase response, individual C/EBP family members are discordinately regulated: C/EBPα levels fall, whereas another C/EBP family member termed nuclear factor IL6 (NF-IL6), is induced. We investigated the interaction between the two acute-phase induced APRE-binding proteins: NF-κB and NF-IL6. Both proteins bind to overlapping nucleotides in a mutually exclusive fashion with similar affinities for the APRE. NF-IL6, a less potent transactivator, attenuates NF-κB mediated transcription late in the evolution of the acute-phase response. These observations argue for a temporal model of sequentially-expressed transcription factors occupying the APRE during the evolution of the inflammatory process.

Inducible hepatic expression of angiotensinogen, the precursor of the potent vasopressor angiotensin II, is activated by steroid hormones [1–3] and acute-phase inflammation [4–6]. Nuclear run-on and gene transfer studies indicate that increases in angiotensinogen expression in response to these stimuli occurs at the transcriptional level.

Using gene transfer studies we have previously reported the *cis*-acting regulatory elements that mediate glucocorticoid activation of the angiotensinogen promoter [2, 7, 8]. The two identified glucocorticoid response elements include GRE I, a consensus 15 base pair element containing the sequence 5′-AGAACATTTT-GTTTC-3′ between nt −584 to −570, and GRE II, a GRE 'half site' containing the sequence 5′-AGAACA-3′ between nt −477 to −472. Site-directed mutations indicate that GRE I is essential for glucocorticoid activation. In contrast, mutation of GRE II attenuates, but does not abolish, steroid activation. Individually, each element confers glucocorticoid induction onto a heterologous promoter, demonstrating enhancer activity. That the glucocorticoid effect is dose-dependent, saturable and antagonized by the antiglucocorticoid RU486 argues that the transcriptional effect is mediated by the glucocorticoid receptor. Indeed, both GREs are recognized by recombinant glucocorticoid receptor by gel shift analysis. This hierarchic nature of GREs within the angiotensinogen gene is similar to arrangements in other hepatic genes, such as the tyrosine aminotransferase gene [9].

During these studies, we also identified a critical *cis* element located between nt −531 and −557, termed the acute-phase response element (APRE), that mediates cytokine induction of angiotensinogen gene expression. The APRE contains the sequence 5′-AGTTGGGATTTCCAACC-3′ and is recognized by two different classes of DNA-binding proteins. Each class is itself regulated in abundance as a function of acute-phase inflammation.

Results and Discussion

Two classes of transcription factors bind the APRE

Identification of the DNA-binding proteins that interact with the APRE was approached by gel mobility shift assays of purified nuclear proteins harvested from lipopolysaccharide (LPS)-stimulated rat liver. By ion exchange and gel filtration chromatography, an LPS-inducible protein, termed BPi, could be fractionated from a family of heat stable nuclear proteins termed BPc [5].

We demonstrated that the BPi proteins includes members of the nuclear factor-κB (NF-κB) family. The NF-κB/Rel/Dorsal (NRD) transcription factors constitute a family by virtue of containing a highly conserved DNA-binding Rel homology domain in their N-termini [10]. NF-κB members include the 50 kDa protein termed p50, a DNA-binding subunit, and a 65 kDa protein, termed p65, that contains weak DNA-binding activity and is a potent transactivator. NF-κB p65:p50 heterodimers are sequestered in the cytoplasm via interaction with an inhibitor termed IkB. Upon cytokine stimulation of hepatocytes, the NF-κB p65:p50 heterodimer is released from IkB binding and enters the nucleus where it binds the APRE and stimulates transcription. Evidence that BPi is NF-κB includes: cross competition of BPi-APRE binding using NF-κB containing oligonucleotide probes, similar methylation interference patterns of BPi and purified NF-κB from bovine spleen, and detection of sequestered BPi DNA-binding activity in the cytoplasm of unstimulated hepatocytes. Although we used UV cross linking to couple the NF-κB p50 protein subunit to the APRE, demonstration that NF-κB p65,

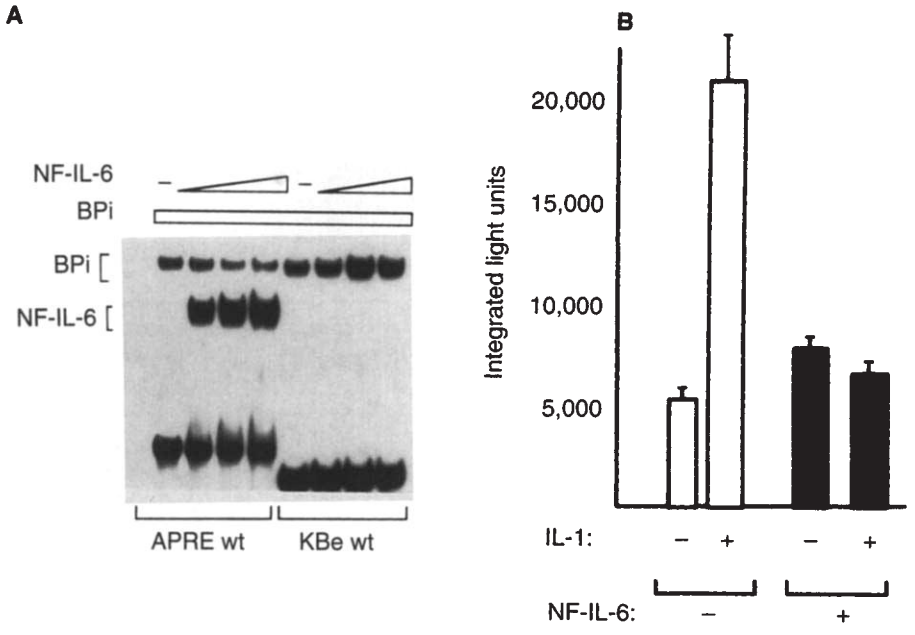

Fig. 1. (A) *NF-IL6 competes with NF-κB (BPi) for binding the angiotensinogen APRE.* Purified NF-κB (BPi) prepared from LPS-stimulated rat hepatocytes was incubated with either radiolabeled APRE (**left**) or NF-κB oligonucleotides in the presence of increasing amounts of NF-IL6. After binding, reactions were analyzed by nondenaturing PAGE and exposed to autoradiography. Location of complexes produced by BPi and NF-IL6 are indicated. With increasing amounts of NF-IL6, the abundance of BPi complexes is diminished only on the APRE DNA template, indicating DNA-specific competition for binding. (**Right**). Overexpression of NF-IL6 blocks NF-κB mediated transcription. HepG2 cells were transiently transfected with angiotensinogen luciferase reporters in the absence or presence of NF-IL6 expression vectors. After 40 hours of transfection, cells in the indicated lanes were stimulated with recombinant IL-1 for 4 hours. Luciferase reporter activity was measured and normalized to internal control alkaline phosphatase reporter activity. NF-IL6 expression attenuates the IL-1 activated, NF-κB mediated transcription of the angiotensinogen APRE.

the potent transactivating subunit of the NF-κB family, interacts with the APRE has been lacking.

NF-κB p65 subunit transactivates the APRE

Transient transfection-overexpression assays are used as sensitive measures to determine whether a cloned transcription factor can regulate the activity of a test enhancer [11]. Transfection of a multimeric APRE-driven luciferase reporter plasmid in the presence of a eukaryotic expression vector lacking or containing coding sequences for the human p65 subunit was performed in the human HepG2 hepatoblastoma cell line. Luciferase activity in transfectants containing the p65 expression vector was increased in a dose-dependent fashion to a maximum of 50-fold stimulation. These data, taken in combination with the ability of p65 antibodies to supershift the TNF-inducible BPi DNA-binding complexes, argues that NF-κB p65 binds and transactivates the APRE *in vivo*. These data strengthen the premise that BPi is indeed NF-κB.

Nuclear Factor-IL6 also binds and transactivates the APRE

The APRE-binding heat-stable nuclear proteins termed BPc were identified to be members of the CCAAT-box/Enhancer Binding Protein (C/EBP) family by the following criteria: ability to cross-compete BPc-APRE binding with C/EBP binding sites, ability of BPc to heterodimerize with recombinant C/EBP peptides in solution, and the observation that anti-C/EBP antiserum cross-reacts with BPc peptides [5].

C/EBP proteins are nuclear DNA-binding proteins containing a highly conserved basic domain-leucine zipper (bZIP) motif. Although initially termed BPc for Binding Proteins constitutive, we subsequently demonstrated that individual C/EBP family members are regulated by acute-phase inflammation. One C/EBP family member in particular, termed nuclear factor-IL6 (NF-IL6, also referred to as C/EBPβ, IL6-DBP, CRP-2, and AGP-EBP), is the major APRE binding protein within eight hours after induction of the acute-phase response *in vivo*.

To study the interaction of NF-IL6 with APRE-DNA, we achieved high levels of expression of recombinant NF-IL6 using an *E. coli* T7 promoter/polymerase-based expression system [12]. Using homogeneous recombinant NF-IL6, we observed that NF-IL6 binds to the APRE in a sequence-specific fashion by DNase I and gel mobility shift analyses. Transient transfection of trimeric APRE binding sites linked to a minimal angiotensinogen promoter-luciferase reporter in the presence of a eukaryotic expression vector for NF-IL6 produces a 40-fold activation of luciferase reporter activity. These data indicate that NF-IL6, too, regulates APRE enhancer activity.

Distinct temporal profiles for NF-κB and NF-IL6 induction

Both NF-κB and NF-IL6, distinct DNA-binding proteins from unrelated transcription factor families, are induced by acute-phase inflammation. However, the temporal profile for their appearance is distinct. NF-κB, a preformed DNA-binding protein sequestered in the cytoplasm of unstimulated hepatocytes, is induced in the nucleus within two hours after induction of inflammation. By contrast, NF-IL6 is found at low levels in unstimulated hepatic nuclei and its levels peak at eight hours after initiation of inflammation. During the evolution of the acute-phase response, both NF-κB and NF-IL6 reside in the nucleus. What is the mechanism of their interaction on the APRE and are there transcriptional consequences?

NF-IL6 competitively displaces NF-κB from the APRE

Investigation of the interaction of NF-κB and NF-IL6 was approached using purified BPi and recombinant NF-IL6 in gel shift assays. In the assay shown in Figure 1, increasing amounts of NF-IL6 was added to the APRE probe in the presence of a constant amount of BPi (NF-κB) protein. With increasing concentrations of NF-IL6, competition for the APRE was observed *in vitro*, but not observed when equal concentrations of recombinant protein were incubated with a DNA probe that binds only NF-κB. These results indicate that NF-κB and NF-IL6 occupy the APRE in a mutually exclusive fashion. To determine whether this

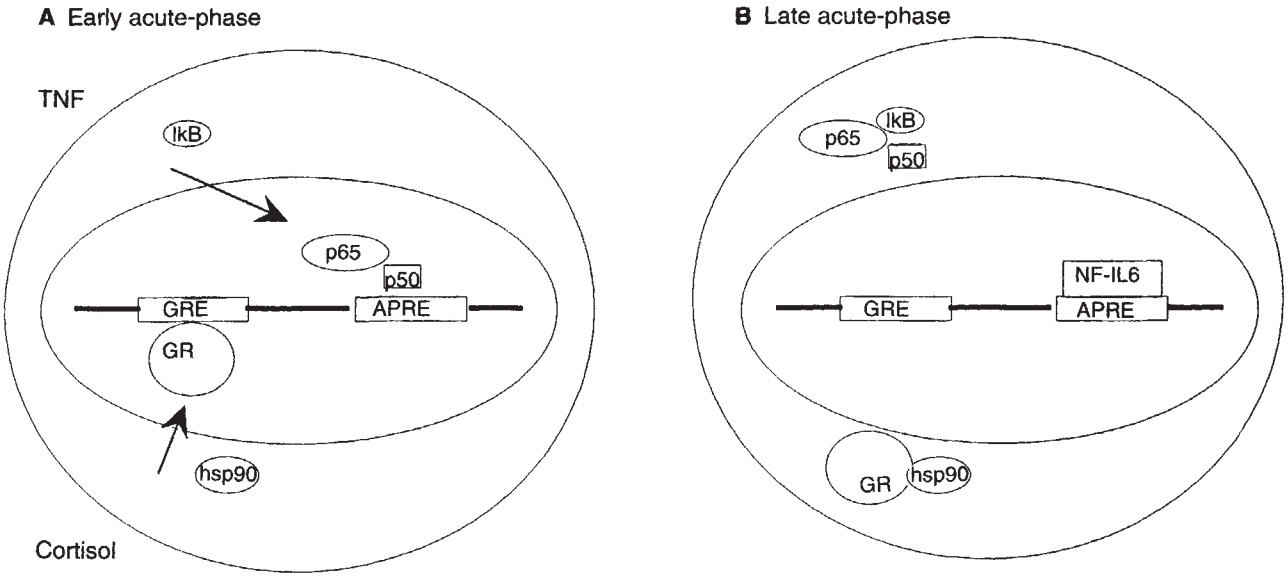

Fig. 2. *Sequential transcription factors occupy the APRE during the evolution of the acute-phase response.* A schematic diagram of hepatocyte early (1–2 hrs) in the evolution of the acute-phase response on the left, and later (8 hrs) on the right. Immediately inducible glucocorticoid receptor (GR) and NF-κB proteins occupy the GRE and the APRE, respectively. Later, NF-IL6 de novo synthesis makes this protein the most abundant APRE binding protein. By its ability to displace the more potent NF-κB, NF-IL6 occupies the APRE, and by virtue of being a less potent activator results in an inhibition of further cytokine activation.

competition *in vitro* results in distinct transcriptional events on the native angiotensinogen promoter *in vivo*, transient expression assays were performed in NF-IL6 deficient HepG2 cells. In the absence of NF-IL6, NF-κB transactivates the native angiotensinogen promoter in response to IL-1. In contrast, when NF-IL6 is present, NF-κB action is blocked (Fig. 2).

Conclusions

These observations argue that during the evolution of the inflammatory response, a sequential pattern of transcription factors occupy the APRE. That the abundance of the NF-κB and NF-IL6 transcription factors are regulated in a tissue-restricted pattern may result in a distinct pattern for angiotensinogen induction in non-hepatic tissues. Moreover, these observations suggest that the angiotensinogen gene can be rendered inactive to cytokine stimulation by prior stimuli that increase the abundance of NF-IL6, and provide one mechanism to terminate NF-κB mediated transcription of the angiotensinogen gene.

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